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(54) Title: METHODS AND COMPOSITIONS FOR SEQUENCE-SPECIFIC HYBRIDIZATION OF RNA BY 2'-5' OLIGONUCLEOTIDES

(57) Abstract

A 2'-5' oligonucleotide is hybridized to complementary nucleic acid which may be RNA or duplex DNA. The 2'-5' oligonucleotide is a methylphosphonate, a phosphorothioate or, especially preferred, a phosphodiester.

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METHODS AND COMPOSITIONS FOR SEQUENCE-SPECIFIC HYBRIDIZATION OF RNA BY 2'-5' OLIGONUCLEOTIDES

FIELD OF THE INVENTION

It is the primary objective of this invention to provide 2'-5' oligonucleotides as agents to determine gene expression in a sequence-specific manner. In particular, this invention is directed to the determination of mRNA levels via an antisense strategy using 2'-5' oligonucleotides i.e. nucleotides connected by 2'-5' intersugar linkages. More particularly this invention relates to the use of oligonucleotides having 2'-5' internucleo-tide linkages to hybridize to complementary mRNAs or pre-mRNAs. More particularly this invention relates to the use of (2'-5')oligo-3'-deoxynucleotides containing a natural phosphodiester backbone to selectively hybridize complementary RNA. This invention also relates to the synthesis of (2'-5')oligo-3'-deoxynucleotides via a solid-phase phosphoramidite approach.

BACKGROUND OF THE INVENTION

Sequence specific interactions between nucleic acids by Watson-Crick base pairing, or between nucleic acids and proteins proceed by well-defined recognition rules which govern all steps of gene expression. In principle, specific interference with any such event would provide a means to control cellular or viral gene expression. The antisense strategy has been used in a pharmacological manner to block the expression of various genes (for reviews see: (i) Uhlman,

E.; P yman, A. *Chem. Rev.* **1990**, 90, 543. (ii) Stein, C.A.; Cohen, J.A. *Cancer Res.* **1988**, 48, 2659. (iii) Matteucci, M.D.; Bischofberger, N. *Annu. Rep. Med. Ch m.* **1991**, 26, 287. (iv) Miller, P.S.; Ts'o, P.O.P. *Annu. Rep. Med. Chem.* **1988**, 23, 295. (v) Neckers, L.; Whitesell, L.; Rosolen, A; Geselowitz, D.A. *Critical Revs. Oncogenesis* **1992**, 3, 175. (vi) *Gene Regulation: Biology of Antisense RNA and DNA, Volume 1,* Erickson, R.P.; Izant, J.G., Eds.; Raven Press: New York 1992). Antisense oligonucleotides are short single-stranded DNA or RNA fragments whose nucleotide sequence is complementary to a specific sequence within the target mRNA. The antisense oligonucleotide hybridizes to the mRNA which thereby inhibits gene expression by possibly blocking processing, transport, or translation of the sense mRNA. The inhibition of translation observed may also be due to cleavage of the mRNA by ribonuclease H (RNase H), an enzyme found in the nuclei of mammalian cells that is able to hydrolyze the RNA strand of an RNA-DNA hybrid. Endogenous RNase H-like activity may play a role in the specific inhibiting properties of antisense oligonucleotides observed in cultured cells.

Examples of the success of the antisense strategy using oligonucleotides include inhibition of Herpes simplex virus replication (Kulka, M.; Smith, C.C.; Aurelian, L.; Fishelevich, R.; Meade, K.; Miller, P.; Ts'o, P.O.P. *Proc. Natl. Acad. Sci. USA* **1989**, 86, 6868) and blocking viral protein synthesis of HIV-1 (Agrawal, S.; Ikeuchi, T.; Sun, D.; Sarin, P.S.; Konepka, A.; Maizel, T.; Zamecnik, P.C. *Proc. Natl. Acad. Sci. USA* **1989**, 86, 7790). Antisense

oligonucleotides have also been shown to inhibit the expression of specific onc genes in cell culture, such as c-myc (Wickstrom, E.L.; Bacon, T.A.; Gonzalez, A.; Freeman, D.L.; Lyman, G.H.; Wickstrom, E. *Proc. Natl. Acad. Sci. USA* **1988**, 85, 1028) and c-myb (Gewirtz, A.M.; Calabretta, B. *Science* **1988**, 242, 1303).

The goal in the development of antisense oligonucleotides is to inhibit specific gene expression in intact cells. The desired properties of antisense oligonucleotide and oligodeoxynucleotides includes stability against nucleases, membrane permeability and selective inhibition of gene expression. Unmodified phosphodiester antisense oligodeoxynucleotides and antisense RNA have been shown to inhibit translation of targeted mRNA but are susceptible to rapid degradation by nucleases within the cells as well as in mammalian sera. Therefore, much effort has been made to synthesize oligonucleotide analogs with modified internucleotide linkages e.g., phosphorothioate, (Eckstein, F.; *Annu. Rev. Biochem.* **1985**, 54, 367) methylphosphonate (Ts'o, P.O.P.; Miller, P.S.; Aurelian, L.; Blake, K.R.; Murakami, A.; Agris, C.; Blake, K.R.; Lin, S.-B.; Lee, B.L.; Smith, C.C. *Ann. N.Y. Acad. Sci.* **1988**, 507, 220) phosphorodithioate, (Brill, W.K.-D.; Tang, J.-Y.; Ma, Y.-X.; Caruthers, M.H. *J. Am. Chem. Soc.* **1989**, 111, 2321) ethylphosphotriester, (Miller, P.S.; Chandrasegaran, S.; Dow, D.L.; Pulford, S.M.; Kan, L.S. *Biochemistry* **1982**, 21, 5468) and phosphoramidate (Froehler, B.; Ng, P.; Matteucci, M. *Nucleic Acids Res.* **1988**, 16, 4831). The majority of the

modifications are directed primarily towards the sugar-phosphate backbone and usually involve a minimal change of ligands around the phosphorous atom to prevent distortion in the geometry of the internucleotide bond and thereby maintain fidelity of oligomer binding while enhancing stability and nuclease resistance. There is as yet no universally applicable oligonucleotide structure to serve as an antisense effector. Unmodified phosphodiester oligodeoxynucleotides offer the advantages of good solubility, efficient and stable hybridization and activation of RNase H, but suffer from poor biological stability and poor cellular uptake. Methylphosphonate oligonucleotide analogs are poorly soluble and are unable to direct cleavage of RNA by RNase H. Phosphorothioates are able to survive longer than unmodified oligonucleotides in cells and media due to their nuclease resistance, however, they enter cells more slowly, possibly a result of stronger binding to one or more cell-surface receptors or other proteins (Loke, S.L.; Stein, C.A.; Zhang, X.H.; Mori, K.; Nakanishi, M.; Subasinghe, C.; Cohen, J.S.; Neckers, L.M. *Proc. Natl. Acad. Sci. USA* **1989**, 86, 3474). Phosphorothioates also suffer from the disadvantages of toxicity and non-specific inhibition of protein and DNA synthesis at concentrations which are near those required for sequence- specific effects. Phosphorothioate and methylphosphonate backbone-modified oligodeoxynucleotides exist as diasteromeric mixtures and form less stable hybrids than normal phosphodiester oligonucleotides (Freier, S.M.; Lima, W.F.; Sanghvi, Y.S.; Vickers, T.; Zounes, M.; Cook,

P.D.; Ecker, D.J. in *Gene Regulation: Biology of Antisense RNA and DNA, Volume 1,* pp.95-107; Erikson, R.P.; Izant, J.G., Eds.; Raven Press: New York 1992) (Miller, P.S.; Yano, J.; Yano, E.; Carroll, C.; Jayaraman, K.; Ts'o, P.O.P. *Biochemistry* **1979**, 18, 5134). Chirality may also be important in the case of phosphorothioates in directing RNase H activation of the phosphorothioate oligodeoxynucleotide-RNA heteroduplex. Agrawal has reported that phosphodiester-linked oligodeoxynucleotides are more efficient than the corresponding phosphorothioate analogs with respect to human RNase H activity (Agrawal, S.; Mayrand, S.H.; Zamecnik, P.; Pederson, T. *Proc. Natl. Acad. Sci. USA* **1990**, 87, 1401). The ability to serve as a template for RNase H may have therapeutic value by mediating, or at least enhancing the antisense effect relative to oligonucleotides that are unable to activate RNase H. However the exact role of an RNase H activity in intact cells remains to be ascertained.

The problems arising for example, from chirality, steric hindrance, or hydrophobicity as well as the potential risk of toxicity and antigenicity in vivo, prompted us to consider oligodeoxynucleotides which are constitutional isomers of biological DNA differing only in bond connectivity. One possible approach to modifying an oligonucleotide to generate a constitutional DNA isomer involves the alteration of the sugar moiety. The reversion of the configuration of the 1' carbon atom of the sugar residue results in α-oligonucleotide analogs (Morvan, F.; Rayner, B.; Imbach, J.-L.; Chang, D.K.; Lown, J.W. *Nucleic Acids Res.* **1986**, 14, 5019) (Morvan, F.;

Rayner, B.; Imbach, J.-L.; Lee, M.; Hartley, J.A.; Chang, D.K.; Lown, J.W. *Nucleic Acids Res.* **1987**, 15, 7027) (Imbach, J.-L.; Rayner, B.; Morvan, F. *Nucleosides & Nucleotides* **1989**, 8, 627). Oligo-α-deoxynucleotides are nuclease resistant and form stable double helices with complementary DNA or RNA sequences (Gagnor, C.; Bertrand, J.R.; Theret, S.; Lemaitre, M.; Morvan, F.; Rayner, B.; Malvey, C.; Lebleu, B.; Imbach, J.-L.; Paoletti, C. *Nucleic Acids Res.* **1987**, 15, 10419) (Cazenave, C.; Chevrier, M.; Thuong, N.T.; Helene, C. *Nucleic Acids Res.* **1987**, 15, 10507). They are capable of antisense inhibition of β-globin mRNA translation via an RNase H independent mechanism (Boiziau, C.; Kurfurst, R.; Cazenave, C.; Roig, V.; Thuong, N.T. *Nucleic Acids Res.* **1991**, 19, 1113). Similarly, Beaucage has recently reported that alternating α,β-oligothymidylates with alternating (3'-5')- and (5'-5')-internucleotide phosphodiester linkages exhibit enhanced nuclease resistance and hybridize with satisfactory affinity to complementary DNA and RNA (Koga, M.; Moore, M.F.; Beaucage, S.L. *J. Org. Chem.* **1991**, 12, 3757).

In some instances substitution of 2'-deoxy-β-D-ribofuranose by an isomeric sugar residue generates an oligodeoxynucleotide that exhibits selective hybridization to DNA and RNA complements. A pentadecanucleotide prepared from 1-α-D-arabinofuranosylthymine hybridizes with some selectivtity to complementary RNA rather than DNA (Adams, A.D.; Petrie, C.R.; Meyer, R.B. *Nucleic Acids Res.* **1991**, 19,

3647). Another sugar modification which generates a constitutional DNA isomer is the replacement of the 2'-deoxy-D-ribose backbone by 2'-deoxy-L-*erythro*-pentose to give enantio-DNA. Enantio-DNA (L-dA_6) has been shown to be resistant to bovine spleen phosphodiesterase and binds complementary RNA preferentially to complementary DNA (Shizuyoshi, F.; Shudo, K. *J. Am. Chem. Soc.* **1990**, 112, 7436).

The 2'-5' internucleotide linkages of oligoadenylates (2'-5')A_n, represent unique examples of naturally occurring constitutional RNA isomers. The (2'-5')A_n oligomers have been detected in a variety of cells and tissues including L1210 cells and human lymphocyctes (Cailla, H.; LeBorne De Kaouel, C.; Roux, D.; Delage, M.; Marti, J. *Proc. Natl. Acad. Sci. USA* **1982**, 79, 4742). The (2'-5')A_n has been suspected to be involved in regulation of cell growth and differentiation and in the antiviral mechanism of interferon (Wells, M.; Mallucci, L. *Exp. Cell Res.* **1985**, 159, 27). In the (2'-5')A pathway interferon and double-stranded RNA activate an enzyme, (2'-5')-oligoadenylate synthetase, to catalyze the formation of oligoadenylates from ATP linked 2'-5' rather than by the usual 3'-5' phosphodiester bonds. The oligoadenylates vary in length from two to fifteen residues. The di-, tri- and tetraadenylates are the most abundant and the amounts of larger oligoadenylates diminish with increasing chain lengths (Samanta, H.; Dougherty, J.P.; Lengyel, P. *J. Biol. Chem.* **1980**, 255, 9807). The (2'-5')A_n subsequently binds and activates an endoribonuclease (RNase L) which is responsible

for the nonspecific cleavage of messenger and ribosomal RNAs and thereby inhibits protein synthesis in intact cell systems (Farrell, P.J.; Sen, G.G.; Dubois, M.F.; Ratner, L.; Slattery, R.E.; Lengyel, P. *Proc. Natl. Acad. Sci. USA* **1978**, 75, 5893). Double-stranded RNA is not cleaved during the process (Ratner, L.; Sen, G.C.; Brown, G.E.; Lebleu, B.; Kawakita, M.;Cabrer, B.; Slattery, E.; Lengyel, P. *Eur. J. Biochem.* **1977**, 79, 565).

The biological activity of (2'-5')-oligoadenylates is rapidly lost due to (i) cleavage of the 2'-5' internucleotide bond by a specific 2'-5'-phosphodiesterase which begins from the 2' end and degrades in a processive manner and (ii) one or several phosphatases which dephosphorylate $(2'-5')A_n$ to its core congener. This has led to the synthesis of a plethora of structurally modified $(2'-5')A_n$ analogs designed to improve cellular stability and uptake as well as better characterize its binding and activation of RNase L. For example, the half-life of $(2'-5')A_3$ in tissue culture is three hours; however the replacement of the 3' hydroxyl group of the adenosine moieties of $(2'-5')A_n$ by hydrogen atoms (i.e., cordycepin analogs) retains the properties of achirality and increases the half-life at the internucleotide linkages to seventeen hours against 2'-phosphodiesterase and cellular nuclease activity (Kariko, K.; Reichenbach, N.L.; Suhadolnik, R.J.; Charabula, R.; Pfleiderer, W. *Nucleosides & Nucleotides* **1987**, 6, 497).

The (2'-5')oligo-3'-deoxyadenylates are nontoxic to cells and exhibit a broad spectrum of biological activities (Kariko,

K.; Reichenbach, N.L.; Suhadolnik, R.J.; Charubala, R.; Pfleiderer, W. *Nucleosides & Nucleotides* **1987**, 6, 497) (Torrence, P.F.; Imai, L.; Jamoulle, J.C.; Lesiak, K. *Ch m. Scripta* **1986**, 26, 191). Cordycepin trimer and its 5'-monophosphorylated analog fail to activate RNase L but do inhibit to some extent HIV-1 reverse transcriptase in vitro with no cell toxicity at a concentration of 62.5 mM (Sawai, H.; Imai, J.; Lesiak, K.; Johnston, M.I.; Torrence, P.F. *J. Biol. Chem.* **1983**, 258, 1671). Furthermore, it appears unlikely that under experimental conditions, the cordycepin trimer serves as a prodrug of cordycepin which has no anti-HIV-1 activity in vitro (Montefiori, D.C.; Sobol, R.W.; Li, S.W.; Reichenbach, N.L.; Suhadolnik, R.J.; Charbula, R.; Pfleiderer, W.; Modliszewski, A.; Robinson, W.E.; Mitchell, W.M. *Proc. Natl. Acad. Sci. USA* **1989**, 86, 7191).

Three adenosine monophosphate residues linked 2'-5' and a 5'-phosphorylated moiety are required for binding RNase L. For activation of RNase L, a 5'-di- or 5'-triphosphate is required (Kariko, K.; Reichenbach, N.L.; Suhadolnik, R.J.; Charubala, R.; Pfleiderer, W. *Nucleosides & Nucleotides* **1987**, 6, 497). When the 2'-5' phosphodiester bond(s) of a 2'-5'A trimer are replaced with 3'-5' phosphodiester linkages a 10^5-fold decrease in inhibition of translation and a 13,000-fold decrease in ability to bind to RNase L are observed (Lesiak, K.; Imai, J.; Floyd-Smith, G.; Torrence, P.F. *J. Biol. Chem.* **1980**, 258, 13082). There is no detectable 5'-rephosphorylation of the (2'-5')-3'-dA_n core of trichloroacetic acid (TCA)-soluble cytoplasmic extracts of

lymphocytes and lymphoblasts (Suhadolnik, R.J.; Doetsch, P.W.; Devash, Y.; Henders n, E.E.; Charubala, R.; Pfleiderer, W. *Nucleosides & Nucleotides* **1983**, 2, 351).

It is unlikely that long nonphosphorylated (2'-5')-3'-dA_n oligomers (n>4) will bind and activate RNase L or inhibit protein synthesis, (Lee, C.; Suhadolnik, R.J. *FEBS Lett.* **1983**, 1, 205) however, they may have antimitogenic properties in intact cells (*Nucleosides & Nucleotides* **1983**, 2, 351). Furthermore substitution of one the adenosine moieties of a (2'-5')A trimer with uridine results in a marked decrease in binding and activation of RNase L (Kitade, Y.; Alster, D.K.; Pabuccuoglu, A.; Torrence, P.F. *Bioorg. Chem.* **1991**, 19, 283).

Based on these highly defined structural requirements the interaction of (2'-5')oligo-3'-deoxynucleotides with RNase L appears selective for adenosine residues of $n \leq 4$ bases. Thus, it would not be expected that mixed base sequences of longer oligomers (~21 mers), commonly used as modulators of gene expression, containing 3'-deoxy-(2'-5') internucleotide linkages would non-specifically inhibit protein synthesis by the (2'-5')A system.

Furthermore, whileas all structural modifications of the 2',5'-oligonucleotide system done till date were designed to ultimately improve on their biological activity for protein targets (activation of RNase L and 2',5'-oligoadenylate synthetase etc.) none of the studies were designed to determine the binding efficiency of these oligonucleotides to nucleic acid targets (DNA and RNA).

In order for a 2'-5' oligonucleotide to serve as an

effective analog to determine gene expression it must bind with complementary base sequences in the target nucleic acid. Theoretical studies on the stability of helices with 2'-5' linked nucleic acids have led to conflicting predictions (Anukanth, A.; Pannuswamy, P.K. *Biopolymers* **1986**, 25, 729; Srinivasan, A.R.; Olson, W.K. *Nucleic Acids Res.* **1986**, 14, 5461). Conformational analysis of dimer and trimer units of $(2'-5')A_n$ (n=2,3) by nuclear magnetic resonance and circular dichroism studies indicate that the 2'-5' nucleotides show a stronger tendency to base stack even at elevated temperatures than the corresponding 3'-5' ribonucleotides (Doornbos, J.; Den Hartog, J.A.J.; van Boom, J.H.; Altona, C. *Eur. J. Biochem.* **1981**, 116, 403; Johnston, M.I.; Torrence, P.F. in *Interferons, Volume* 3, pp.189-298; Friedman, R.M., Ed.; Elsevier: Amsterdam, 1984; Torrence, P.F. in *Biological Response Modifiers - New Approaches to Disease Intervention*, pp.77-105; Torrence, P.F., Ed. Academic: New York, 1985; Lengyl, P. *Annu. Rev. Biochem.* **1982**, 51, 251). Recently Turner has provided experimental evidence that complementary decamers of 2'-5' linked oligoribonucleotides can form antiparallel duplexes by Watson-Crick hydrogen bonding (Kierzek, R.; He, L.; Turner, D.H. *Nucleic Acids Res.* **1992**, 20, 1685). The overall stability, however, of the 2'-5' duplexes is less than the corresponding 3'-5' duplexes, presumably due to a less favorable enthalpy change for association.

In the 3'-deoxynucleotide series, 2'-5' helices of mixed sequences and homopolymers also weakly strand associate as

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shown by Tm studies and a mobility shift assay (Dougherty, J.P.; Rizzo, C.J. Breslow, R.J. *Am. Ch m. Soc.* 1992, 114, 6254). The association between complementary (2'-5') oligo-3'- deoxynucleotides was shown to improve when uridine was substituted for thymidine (Hashimoto, H.; Switzer, C.J. *Am. Chem. Soc.* 1992, 114, 6255). The complementary (2'-5')oligo-3'-deoxynucleotides dA_{12} and dU_{12} exhibit a Tm of 22.8°C versus 40.8°C for the (3'-5')-linked DNA helix at high salt (Hashimoto, H.; Switzer, C.J. *Am. Chem. Soc.* 1992, 114, 6255).

The attractive features of conformational flexibility, high biological stability, low cell toxicity and the natural phosphodiester structure suggests that (2'-5')oligo-3'-deoxynucleotides represent a novel backbone structure to serve as an effective antisense inhibitor of gene expression in mammalian cells. An essential requirement in the antisense approach is that an oligonucleotide or its analog recognize and bind tightly to its complementary RNA sequence. The possibility of a 2'-5' oligomer associating with complementary 3'-5' nucleic acids has not been reported. It is the purpose of this invention to provide 2'-5' oligonucleotides for use in therapies for sequence specific inhibition of gene expression via hybridization to complementary mRNA or complementary duplex DNA.

Novel methodologies to evaluate large numbers of oligonucleotides with therapeutic value have recently been reported (Ellington, A.D.; Szostak, J.W. 1992, Nature, 355,

850) (Tuerk, C.; Gold, L. Science 1990, 249, 505) (Ellington, A.D.; Szostak, J.W. Nature, 1990, 346, 818). An experimental procedure called SELEX (systematic evolution of ligands by exponential enrichment) has been described as a general way to study protein-nucleic acid interactions (Tuerk, C.; Gold, L. Science 1990, 249, 505). In this procedure random pools of oligonucleotides containing approximately 10^{13} different molecular species, each having a different nucleotide sequence are synthesized. These pools are then incubated with the target molecule, and substances that bind with the highest affinity are isolated by physical separation techniques, such as affinity chromatography or filter binding. The isolated pool is then amplified by enzymatic procedures, and the binding, selection and amplification cycles are repeated until the pool is enriched with only those oligonucleotides that have the greatest affinity. This technique allows for the selection of oligonucleotides that, by chance, have the correct three-dimensional structure to bind to a target molecule. In subsequent steps, the high-affinity oligonucleotides are evaluated for their ability to inhibit activity, for example, enzymatic activity of the target to which they bind.

Aptamers having an affinity to large proteins or small organic target structures, can be selected. Thus, high-affinity inhibitors can potentially be found for any extracellular target molecule for which a therapeutic benefit may be derived. Most importantly, aptamer selection steps can be manipulated to screen the aptamer pool by more criteria

than mere affinity for a given target molecule. Thus, other properties that are essential for therapeutic success can be conferred upon the final oligonucleotide.

BRIEF DESCRIPTION OF THE DRAWING

Figure-1. is a schematic drawing for the synthesis of 3'-deoxysugar synthon.

Figure-2. is a schematic drawing for the synthesis of 3'-deoxy pyrimidine building blocks.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method of determining gene expression in an organism which comprises hybridizing at least one 2'-5' oligonucleotide in a sequence specific manner to complementary mRNA of said organism. In a preferred embodiment, the 2'-5' oligonucleotide is from about 8 to about 75 nucleotides in length. In other preferred embodiments the 2'-5' oligonucleotide is a methylphosphonate, a phosphorthioate or, especially preferred, a phosphodiester. In yet other preferred embodiments, the 2'-5' oligonucleotide is chemically modified to increase its stability against nuclease degradation, to enhance its permeability into cells, to increase its binding strength upon hybridization, or to trigger a crosslinking reaction, cleavage reaction or combination thereof with complementary mRNA or complementary duplex DNA.

In another aspect of the invention, a method is provided for treating a disease characterized by undesired protein synthesis, which comprises administering to a human patient in need of such treatment at least one 2'-5' oligonucleotide

having at least one nucleotide unit connected by a 2'-5' linkage wherein the oligonucleotide is substantially complementary to at least a portion of a sequence of an mRNA or duplex DNA encoding the undesired protein.

In a further aspect, a method is provided for treating an agricultural or horticultural disease characterized by undesired protein synthesis, which comprises administering to a plant in need of such treatment, at least one 2'-5' oligonucleotide having at least one nucleotide unit connected by a 2'-5' linkage wherein said oligonucleotide is substantially complementary to at least a portion of a sequence of an mRNA or duplex DNA encoding the undesired protein.

In yet another aspect, the invention provides a pharmaceutical composition which comprise a carrier and a therapeutically effective amount of at least one 2'-5' oligonucleotide having a nucleotide sequence substantially complementary to at least a portion of the mRNA transcript or duplex DNA encoding a target protein so as to block expression of the target protein.

In another aspect of the invention, there is provided a high affinity ligand that includes at least one 2'-5' oligonucleotide containing about 8 to 75 nucleotides wherein the ligand binds to DNA binding proteins. In a preferred embodiment, the ligand is capable of binding to a small molecule of molecular weight less than 5000.

For the purpose of this specification and appended claims, all references made herein to the term "2'-5'"

includes oligonucleotides formed from naturally occurring bases, sugars and phosphate linkages whereby the linkages between nucleotides occur from the 2' end of the sugar residue to the 5' end of the next sugar residue on the polynucleotide chain. In addition chimeric oligonucleotides containing a combination of 3'-5' and 2'-5' internucleotide linkages are also included.

Thus, the invention includes oligonucleotides of the following formula:

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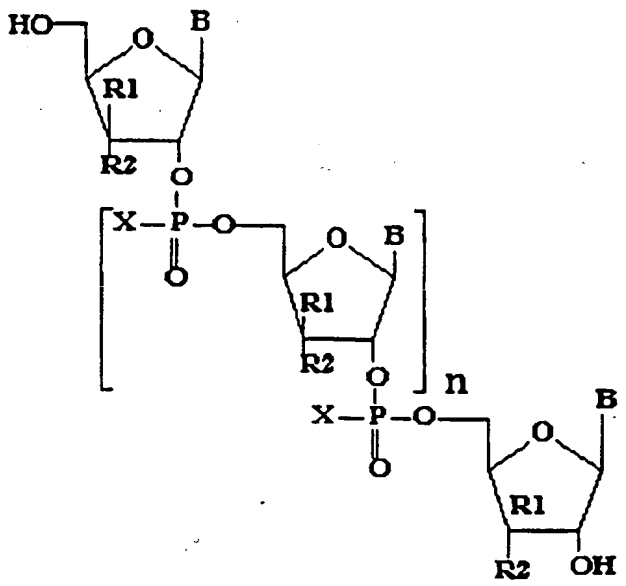


wherein B is a common nucleoside purine or pyrimidine base; R_1 and R_2 are independently nitrogen (azido), hydrogen, alkyl or alkyloxy of from 1 to about 20 carbon atoms, allyl or alkyloxy of from 2 to about 20 carbon atoms or aryl or aryloxy of from 6 to about 20 carbon atoms; and X represents an oxygen atom, a sulfur atom, alkyl, allyl, aryl, alkoxy, allyloxy, aryloxy, alkylamine, allylamine, arylamine, S-alkyl, S-allyl, or S-aryl.

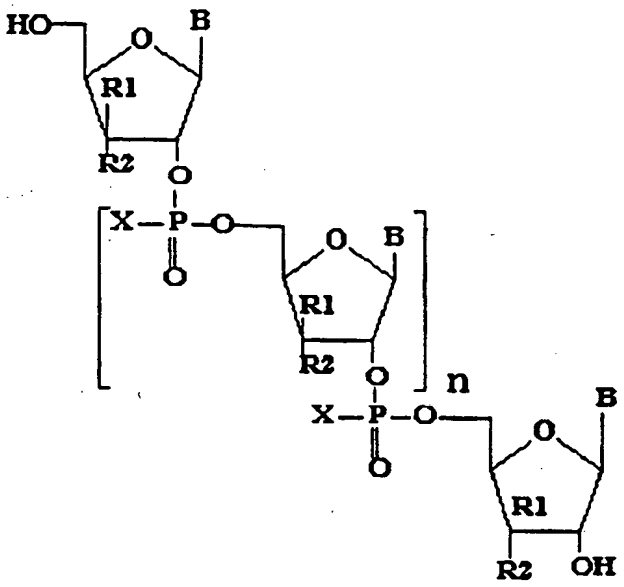
"Dephospho" internucleotide analogs or nonphosphate internucleotide linkages wherein the bridging phosphate is replaced by a different group to connect or bridge nucleoside units, would include but are not necessarily limited to siloxane bridges, carbonate bridges, carboxymethyl ester bridges, acetamidate bridges, carbamate bridges or thioether bridges.

Th term "substantially complementary" is used herein to indicate that the oligonucleotide is capable of hybridizing to and forming a stable heteroduplex with its target sequence in the mRNA transcript in vivo.

The term "high affinity ligand" is used herein to refer to a ligand containing at least one oligonucleotide selected by affinity binding to a target molecule.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The present invention relates to pharmaceutical compositions containing nucleotides possessing 2'-5' internucleotide linkages; and the therapeutic use of such 2'-5' oligonucleotides. In accordance with one embodiment of this present invention, a 2'-5' oligonucleotide is hybridized to complementary nucleic acid which may be mRNA or duplex DNA for the purpose of modulating gene expression. The oligonucleotides of this invention may be represented by the following formula:



wherein B is a common nucleoside purine or pyrimidine base such as adenine, guanine, cytosine, thymine, uracil or a substituted purine or pyrimidine base. Such substituted bases include, but are not necessarily limited to 8-azidoadenine, 8-mercaptoadenine, 8-aminoadenine, 6-thioguanine, 8-azaguanine, 5-fluorouracil, and 5-methylcytosine. Natural sugars include β-D-ribofuranose and 3'-deoxy-β-D-ribofuranose. The glycosidic linkage in the oligonucleotide is in the naturally occurring β-anomeric form but also includes the α-anomeric configuration about the glycosidic bond. Oligonucleotides prepared from β-D-ribofuranose are linked from the 2' oxygen of the sugar to the 5' oxygen of the next nucleotide. The R1 and R2 groups at the 3' position of the β-D-ribofuranose are independently hydrogen, hydroxy, alkyl,

allyl, aryl, alkoxy, allyloxy or aryloxy and may include from one to about twenty carbon atoms. The 3' position

may also be an  group wherein R_1 and R_2 are

independently nitrogen (azido), hydrogen, alkyl, allyl or aryl groups containing from one to about twenty carbon atoms. The 2'-5' oligonucleotides may also include modified bases and sugars in part or all of the oligomer. Modified bases and sugars include but are not necessarily limited to derivatized bases, derivatized b-D-ribofuranosyl, 3'-deoxy-β-D-ribofuranosyl, 3'-deoxy-β-L-*erythro*-pentofuranosyl sugars and carbocyclic pentose sugars. X in the formula includes but is not necessarily limited to an oxygen atom (phosphodiester); sulfur atom (phosphorothioate); alkyl, allyl, or aryl of from one to about twenty carbon atoms (phosphonates); alkoxy, allyloxy, or aryloxy of from one to about twenty carbon atoms (phosphotriester); alkylamine, allylamine, or arylamine from one to about twenty carbon atoms (phosphoramidate); S-alkyl, S-allyl, or S-aryl of from one to about twenty carbon atoms (phosphorothioate). Other heteroatom substituents, e.g., N, O, S, may be attached to the carbon atom chains without departing from the spirit of the present invention. The methods used to prepare these derivatives are well known to those skilled in the art. The invention furthermore provides

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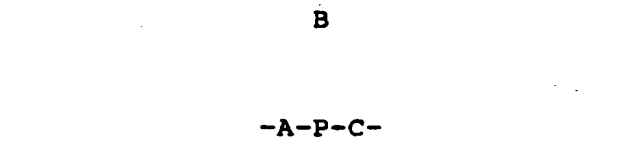
2'-5' linked oligonucleotides containing substitution of either or both of the bridging 5' and 2' oxygen at ms of the phosphate backbone by different heteroatom(s) which include but are not limited to the examples listed in the table below wherein R is hydrogen, alkyl, allyl or an aryl group of from 1 to about twenty carbons. Examples of these types of substitutions are known for oligonucleotides containing 2'-deoxy-(3'-5') internucleotide linkages and may be used for oligodeoxynucleotides joined by 2'-5' linkages by similar chemical means apparent to those skilled in the art (see J. Goodchild *Bioconjugate Chem.* **1990**, 1, 164 and references cited therein).

Modified Internucleoside Phosphates



A	B	C	D
NH	O	O	O
O	O	NH	O
O	S	O	CH_3
O	O	O	Se
O	O	S	O

O	NPr	O	NEt2
O	S	O	NEt2
O	Se	O	NEt2
O	S	O	CH_3
O	S	O	S
O	S	O	NHR
O	S	O	OPr
O	S	O	OEt
S	O	O	O
O	O	OPO_3	O
CH_2	O	O	O
S	O	O	O
S	S	O	O
O	O	S	CH_3

The 2'-5' oligonucleotide is not necessarily limited to linear single-stranded species but also includes oligomers containing secondary structures. Secondary structures may be regarded for the purposes of the present invention as a shape or conformation of the oligonucleotide that include, but are not limited to, circular, stem-loop, or "dumbbell-type" structures for the purpose of enhancing cellular uptake, nuclease resistance and/or improving binding. Secondary structure may be introduced by chemical or enzymatic methods well known in the art. Oligonucleotides transformed to these types of secondary structures may also include portions of nucleotides linked 3'-5'. A portion of the shape may include a sequence of (2'-5')oligo-3'-deoxynucleotides complementary to a target

mRNA.

A preferred group of oligonucl otides useful in this invention are those wherein B is a natural base, especially adenine, guanine, cytosine, thymine and uracil; the sugar moiety is a natural sugar, especially β-D-ribofuranose and 3'-deoxy-β-D-ribofuranose; X is oxygen, sulfur, alkyl, especially methyl, alkoxy especially methoxy or ethoxy; and R1, and R2 are independently hydrogen, hydroxy, NH_2, or alkoxy, especially methoxy. Another preferred modification is sulfur substitution of both non-bridging oxygen atoms. An additional preferred group is substitution of the 5' and 2' bridging oxygen atoms independently by methylene or NH. Most preferably, the oligonucleotides are (2'-5')oligo-3'-deoxynucleotides comprising natural nucleosides and an oxygen phosphodiester backbone. More particularly, the (2'-5')oligo-3'-deoxynucleotides contain a natural phosphodiester backbone substantially complementary to a specific sequence of an mRNA, such that the oligonucleotide can specifically inhibit protein translation.

The cyanoethyl phosphoramidites may be obtained from the corresponding 3'-deoxynucleosides. There are many reported syntheses of 3'-deoxynucleosides including over twenty publications involving syntheses of cordycepin. However, a large number of these syntheses provide low to moderate yields due to poor transformations of the sugar moiety and formation of mixtures of 2' and 3' isomers. Robins has developed a three-step synthesis of cordycepin from readily available adenosine via the ribo-epoxide in 90% overall yield (Hanske,

F.; Robins, M.J. *Tetrahedron Lett.* **1985**, 26, 4295). The key feature in the synthesis is the regioselective ring opening of the ribo-epoxide, 2,3-anhydroadenosine by treatment with lithium triethylborohydride in 98% yield with no detectable amount of 2'-deoxyadenosine. The synthesis is efficient and may be applicable for the conversion of other commercially available ribonucleosides to 3'-deoxynucleosides.

The 3'-deoxynucleosides can be efficiently converted to their fully protected analogs by known methods in the art. A preferred embodiment is acylation via the transient protection method of Jones and dimethoxytritylation of the 5'-hydroxyl to yield the desired protected 3'-deoxynucleoside (Ti, G.S.; Gaffney, B.L.; Jones, R.A. *J. Am. Chem. Soc.* **1982**, 104, 1316).

A portion of the protected 3'-deoxynucleoside can be converted to its 2'-O-succinate and derivatized on lcaa-CPG via the 2' oxygen. In a preferred embodiment, 1-(3-dimethylamino propyl)-3-ethylcarbodiimide (DEC) is used for the conversion. (Pon, R.T,; Usman, N.; Ogilivie, K.K. *Biotechniques* **1988**, 8, 768).

The corresponding cyanoethylphosphoramidite of protected 3'-deoxynucleoside can be prepared by known methods. Preferably, the protected nucleoside is condensed with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (Aldrich) in dichloromethane (25°C, 2h) followed by aqueous work up and flash column chromatography purification (Sinha, N.D.; Biernat, J.; Koster, H. *Nucleic Acids Res.* **1984**, 12, 4539).

The (2'-5')oligo-3'-deoxynucleotides for use in

hybridizing to complementary RNA or complementary duplex DNA are 8-75 nucleotides in length and preferably 8-28 nucleotides in length and may contain different base sequences sufficient to define a unique sequence in the target mRNA transcript. At least 11-15 bases are needed to define a unique sequence in mRNA, where the lower figure refers to oligodeoxynucleotides containing only G and C and the higher figure is the length required for oligodeoxynucleotides containing only A and T (Marcus-Sekura, C.J. *Anal. Biochem.* 1988, 172, 289). Oligodeoxynucleotides of 15-20 bases are more often used since they form stable hybrids with melting temperatures well above 37 °C to ensure that antisense inhibition is elicited, assuming all other factors are favorably addressed. In a preferred embodiment of this invention, a 21 mer (2'-5')oligo-3'-deoxynucleotide is used. Specific oligomers containing a 3'-deoxy-(2'-5') internucleotide linkage may be complementary to regions of a viral DNA, viral RNA, mammalian DNA, or mammalian mRNA. The (2'-5')oligo-3'-deoxynucleotides may serve as therapeutic agents to inhibit synthesis of a specific protein or replication against specific complementary targets by an antisense or an antigene mechanism. The oligodeoxynucleotide may be a natural phosphodiester or a phosphate modified oligomer, e.g. phosphorothioate or methyl phosphonate.

The method of the present invention is effective against all prokaryotes and eukaryotes. Prokaryotes and eukaryotes which can serve as the target for the method of this invention include viruses, bacteria, mycoplasm, single celled

eukaryotes, and animal and human cells. The present method can be used to modify cellular function of living cells in vitro (cell culture), or in vivo where cells form part of the tissue in an animal or human.

While preferred embodiments of the invention have been described herein, it will be evident to those skilled in the art from a reading of the present disclosure that oligodeoxynucleotides containing 2'-5' internucleotide linkages can be used. Modifications to or added substituents to the oligonucleotide directed to the phosphate backbone, 5' and/or 3' terminus, sugar moiety, nucleic acid bases to enhance or confer efficacious properties that would include but are not necessarily limited to solubility, cellular uptake, nuclease resistance, binding strength, a crosslink or cleavage event to irreversibly alter a complementary target are within the scope of the present invention. A complementary target strand may be mRNA or duplex DNA, where mRNA refers to mature RNA and nuclear pre-mRNA.

The stability of (2'-5')oligo-3'-deoxynucleotides of the present invention can be evaluated against the degradative effects of exonucleases and endonucleases by treatment with nucleases. The oligonucleotide is then analyzed by polyacrylamide gel electrophoresis (PAGE). The degradation products are quantitated by laser densitometry.

Various methods of formulation and administration of 2'-5' oligonucleotides are known to those skilled in the medical arts (Avis, K. in *Remington's Pharmaceutical Sciences*, pp.1518-1541; Gennaro, A.R., Ed.; Mack Publishing Company:

Easton, Pa, 1985), the disclosures of which are incorporated herein in their entirety by reference thereto. Such methods of administration may include, but are not limited to, surface application, oral, or parenteral routes, injection into joints, subcutaneous injection, or via sustained release or other pharmaceutical methods of delivery depending on the disease state. Surface application of the compositions of the present invention includes topical application to such surfaces as skin, eyes, lungs, nares, ears, rectum, vagina, stomach, colon and the like.

Appropriate means for parenteral administration include 5% dextrose, normal saline, Ringer's solution and Ringer's lactate. The oligonucleotide may be stored as a lyophilized powder and reconstituted when needed by addition of an appropriate salt solution.

The oligonucleotide may be chemically modified so as to enhance its permeability into cells. Examples of receptor mediated endocytotic systems whereupon chemical conjugation to the oligonucleotide can be used to enhance cellular uptake by targeting a specific cell surface receptor include but are not limited to galactose, mannose, mannose-6-phosphate, transferrin, asialoglycoproteins, water soluble vitamins, e.g. transcobolamin (vitamin B_{12}), biotin, ascorbic acid, folates, any pharmacological agent or analog that mimics the binding of a water soluble vitamin, α-2 macroglobulins, insulin, epidermal growth factor, or attachment to an antibody against a surface protein of the target cell as in the case of the so-called immunotoxins. Chemical conjugation of the

oligonucleotide may also include apolar substituents such as hydrocarbon chains or aromatic groups and polar substituents such as polyamines conjugated to further enhance intracellular uptake. Chemical conjugation of the oligonucleotide to an exogenous molecule may be achieved by covalent, ionic or hydrogen bonding either directly or indirectly by a linking group. Covalent bond formations between the oligonucleotide and an exogenous molecule is the preferred method for conjugation and can be performed via coupling techniques well known in the art.

Furthermore, transmembrane delivery of the oligonucleotide may be achieved by application of protein carriers, antibody carriers, liposomal or other vesicular delivery systems, LIPOFECTIN (TM), electroporation, direct cell fusion, viral carriers, osmotic shock and calcium-phosphate mediated transfection.

The (2'-5')oligo-3'-deoxynucleotides of the present invention hybridize to complementary RNA but not complementary single stranded DNA. The high level of RNA specificity and the strong binding of a (2'-5')oligo-3'-deoxynucleotide to RNA has not been previously reported, and represents a unique feature of this type of internucleotidic motif. Consequently, the (2'-5')oligo-3'-deoxynucleotides can be used to selectively inhibit gene expression by sequence specific hybridization to a target mRNA (antisense). Another possibility is that (2'-5')oligo-3'-deoxynucleotides may inhibit gene expression by binding to a complementary DNA duplex (antigene).

The (2'-5')oligo-3'-deoxynucleotides of the present invention may be prepared by solid phase or solution phase chemistries or enzymatic methods recognized by those skilled in the art. The most preferred method is solid-phase synthesis via cyanoethyl phosphoramidite methodology using standard reagents and protocols. The synthesis may be performed manually via the syringe technique, for example, or on an automated DNA synthesizer (e.g. Milligen 8600) (e.g. Tanaka, T.; Letsinger, R.L. *Nucleic Acids Res.* 1982, 10, 3249).

Diagnosits: 2',5'-connected oligonucleotides of this invention will be useful in diagnostic applications. Since 2',5'-connected oligonucleotides bind preferentially to single stranded RNA over DNA, this property is particularly useful in the determination of various RNA levels in various tissues. This will also enable one skilled in the art to determine the expression of an unwanted or mutant RNA species in tissues by designing a 2', 5'- sequence complementary to the mutant sequence. Tissue samples can be homogenized and then lysed with a detergent. While in a typical experiment, the process would involve initial treatment with DNases to destroy the cellular DNA, isolation of RNA and its fixation on a nylon membrane followed by subsequent treatment with a oligonucleotide probe, superiority of the present invention can be appreciated by the fact that 2',5' probe in the current invention does not require DNase treatment of the samples. The probe being specific for RNA would detect only the complementary RNA in the total cell extract despite of the

presence of complementary DNA sequence in the total extract. Artisans with ordinary skills in this art will appreciate that the invention is n t specifically restricted to the example cited above and that the invention would be generally applicable to diagnostic procedures based on oligonucleotide detection of RNA and modifications thereof.

While the principal objective of the invention is to provide a oligonucleotide probe to determine expression of the proteins in cells, it is hereinafter apparent that such selectivity potential of binding to single stranded RNA only could also enable the oligonucleotide to prevent the normal function of the mRNA. Contemplation of such and other objective utilities for 2',5! oligonucleotides should be thus, obvious to any practitioner with normal skills in this art.

The following examples are provided to more fully illustrate the principles and practices of the invention. The examples are not intended in any way to limit the scope of the invention.

Example-1 Synthesis Of 1,2-Di-O-Acetyl-3-Deoxy-5-O-(4-Methylbenzoyl)-D-α,β-Xylofuranose (Figure-1):

a: Synthesis of 1,2-O-isopropylidene-5-O-(4-methylbenzoyl) α-D-xylofuranose 3:

Commercially available D-xylose 1 was made anhydrous by heating at 70°C overnight over phosphorus pentoxide. Next the sugar (60g, 0.4 mole) dissolved in dry acetone (1.5L) was treated with either 2,2-dimethoxypropane (80 ml) and camphor

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sulfonic acid (9.42 gm, 40 mmol) or with conc. Sulfuric acid (40 ml, 760 mmol) till the solution cleared. Aqueous work up and washing the aqueous layer with dichloromethane (3 x 100 ml) gave after the removal of water, 1,2-O-isopropylidene-a-D-xylofuranose 2 as a syrup. This product (60.2 gm) was co-evaporated with toluene (3x 300 ml) and dried over phosphorus pentoxide. A solution of this crude product in dry pyridine (200 ml) was cooled in a ice-salt bath and toluoyl chloride (43 ml, 320 mmole) in 40 ml of dry pyridine was added dropwise over 1h. The mixture was further stirred for 3 h at room temperature and poured onto ice water (1L). The mixture was extracted in ethyl acetate (3 x 200 ml) and the combined organic phase washed with brine (1x 200 ml), dried (anhydrous sodium sulfate) and concentrated in vacuum to give the crude product. Purification over a column (8 x 100 cm) of 1200 gm silica gel gave the title compound 3 as a solid (64.1 gm) in 73 % overall yield. 1H-NMR (DMSO-d_6): d 7.85, 7.33 d, 4H, tol-H; 5.87, d, 1H, H-1; 5.47, d, 1H, H-2; 4.43, d, 1H, H-4; 4.31, m, 2H, H-5; 4.10, 1H, m, H-3; 2.37, s, 3H, tol-CH_3; 1.38, 1.23, 2s, isopropylidene CH_3.

b: Synthesis of 3-deoxy-1,2-*O*-isopropylidene-5-*O*-(4-methyl benzoyl)-α-D-xylofuranose 5:

A solution of 3 (20 gm, 68.5 mmol) and 4-(dimethylamino)pyridine (22gm, 176 mmol) dissolved in dry acetonitrile (200 ml) was treated dropwise with phenylchlorothionoformate (14 ml, 105 mmol) under a blanket of Argon. The color turned yellow and a solid separated out. Stirring was continued for 1h and solvent removed in vacuum.

The residue was taken up in ethyl acetate (200 ml) and saturated sod. bicarbonate (100 ml). The organic phase was separated and the aqueous phase extracted with ethyl acetate (3x 100 ml). The combined organic phase was washed with brine (1 x 200 ml) dried (anhyd. sod. sulfate) and concentrated in vacuum to give the crude product which was co-evaporated with dry toluene (3 x 10 ml) to give a white solid 4 . ^{1}H-NMR (DMSO-d_6): d 7.88, 7.33 d, 4H, tol-H; 7.45-7.12, m, 5H, Ar-H, 6.03, d, 1H, H-1; 5.66, d, 1H, H-2; 4.89, d, 1H, H-4; 4.68, bs, 1H, H-3; 4.50, m, 2H, H-5, 2.37, s, 3H, 3.75, m, 2-H, H-3; 2.35, s, 3H, tol-CH_3; 1.47, 1.28, 2s, isopropylidene CH_3.

This product was directly dissolved in dry toluene (200 ml). Dry argon was bubbled in this solution for 0.5h followed by the addition of ALBN (1.3 gm, 7.8 mmol) and tributyltinhydride (26 ml, 97.5 mmol). The reaction mixture was heated at 80°C for eight hours under Argon. The reaction mixture was cooled and the solvents removed in vacuo. The residue was partitioned between water (200 ml) and dichloromethane (300 ml). The organic layer was dried over sodium sulfate and concentrated to give after purification on silica gel, 12.5 g (67% yield) of 5 as a thick oil. ^{1}H-NMR (DMSO-d_6): d 7.84, 7.33 d, 4H, tol-H; 5.86, d, 1H, H-1; 5.59, d, 1H, H-2; 4.63, d, 1H, H-4; 4.49, m, 2H, H-5; 2.35, s, 3H, tol-H; 2.25, m, 2-H, H-3; 1.44, 1.21, 2s, isopropylidene CH_3.

Synthesis of 1,2-Di-*O*-acetyl-3-deoxy-5-*O*-(4-methoxy benzoyl)-D-α,β-xylofuranose 7.

Acetonide 5 (12.5 gm, 45.28 mmol) was dissolved in 88% formic acid (550 ml) and stirred for 50°C for 2h. Solvent was removed

in vacuo and the residue co-evaporated with dry toluene (2 x 100 ml) and with dry pyridine (2 x 100 ml). The resulting syrup 6 was dissolved in dry pyridine (500 ml) and acetic anhydride (200 ml, 2.1 mol) added. The reaction mixture was stirred overnight, solvents evaporated and the residue dissolved in ethyl acetate (100 ml) and stirred with sat. sodium bicarbonate solution (100 ml) for 0.5h. The organic phase was separated and the aqueous phase extracted with ethyl acetate (2 x 100 ml). The combined organic extract was washed with brine (1x 200 ml), dried (sod. sulfate) and concentrated to give the crude product which was purified over silica gel (150 gm). Elution with 10% ethyl acetate in light petrol containing 0.2% pyridine afforded after solvent removal, the title compound 7 (10.8 gm, 74.5 % yield). ^{1}H-NMR (DMSO-d_6): d 8.0, 7.24 d, 4H, tol-H; 6.23, d, 1H, H-1; 5.35,5.22, 2d, 1H, H-4; 4.74, m, 1H, H-2; 4.58-4.29, m, 2H, H-5; 2.41, s, 3H, tol-CH_3; 2.28, m, 2-H, H-3; 2.10, 1.98, 2s, isopropylidene CH_3.

Example-2. Syntheses Of Protected Bases.

a: Synthesis of N-acetyl cytosine (C).

Cytosine (2 gm, 18 mmol) was dissolved in dry pyridine-DMF 1:1 (40 ml) and acetic anhydride (4.3 ml, 45 mmol) added. The mixture was stirred at room temperature for 24h. Solvent was removed in vacuo and the residue poured into 100 ml ice-water. The mixture was stirred for 1h and the solid filtered and the residue washed with water. The product was dried over phosphorus pentoxide to give the title compound as white powder (2.75 gm). ^{1}H-NMR (DMSO-d_6) d 11.41, 10.12, 2 bs (D_2O

exchangeable), 1H each, N-H; 7.77, d, 1H, H-6; 2.06, s, 3H, Ac-CH_3.

b: Synthesis of N-benzoyl adenine (A).

Benzoyl chloride (43 ml, 52.4 gm, 0.37 mole) was added dropwise to a suspension of adenine (20 gm, 0.15 mole) in dry pyridine (100 ml). The reaction mixture was refluxed for 2h when it became clear and dark brown in color. The reaction mixture was cooled to room temperature and saturated bicarbonate solution (500 ml) added. The reaction mixture was stirred for 10 mins and extracted into chloroform (500 ml). The chloroform layer was washed with bicarbonate (200 ml) when a solid precipitates out. Cooling in a refrigerator gives 1st crop of product to a combined yield of 23.8 gm (66 % yield). ^{1}H-NMR (DMSO-d_6) d, 8.72, s, 1H, H-8; 8.40, s, 1H, H-2; 8.12-7.52, m, 5H, Ar-H.

c: Synthesis of 2-N-acetyl-6-O-diphenylcarbomyl guanine (G).

Guanine (15.1 gm, 100 mmol) was suspended in dry 1-methyl-2-pyrrolidinone (150 ml) and acetic anhydride (25 ml, 262 mmol) added to it. The reaction mixture was heated at 150°C for 2h. The resulting solution was stirred at room temperature for 12h. The solid was filtered, the solid washed with acetone and dried to give 20.13 gm of 2-N,9-diacetyl guanine. ^{1}H-NMR (DMSO-d_6) d, 12.19, 11.72, 2 bs (D_2O exchangeable), 1H each, N-H; 8.42, s, 1H, H-8; 2.79, 2.19, 2s, 3H each, Ac-CH_3.

The bis acetylated compound (11.76 gm, 50 mmol) was suspended in a mixture of dry pyridine (250 ml) and diisopropyl ethylamine (17.4 ml) followed by a slow addition of

diphenylcarbamoyl chloride. The color changes to orange immediately. The reaction mixture was stirred for 1h followed by addition of 25 ml of ice-cold water. The mixture was stirred for additional 10 mins and the solvent removed in vacuo. The residue was co-evaporated with toluene (3 x 100 ml) and the pink-orange residue taken in 50 % ethanol (600 ml). The reaction mixture was heated at 100°C for 1.5h and kept at room temperature for 24h. The solid was filtered, washed with ethanol and dried to give 18.45 gm of product (95 % yield). ^{1}H-NMR (DMSO-d6)·d, 13.56, bs, (D_2O exchangeable), 1H, imidazole N-H; 10.62 bs, (D_2O exchangeable),1H, N-H; 8.44, s, 1H, H-8; 7.47-7.0, m, 10H, Ar-H; 2.15, s, 3H, Ac-CH_3.

d: Synthesis of 2,6-Bis(acetamido)purine.

2,4-Diaminopurine (15 gm, 100 mmol) was taken in dry pyridine (200 ml) and acetic anhydride (31 ml, 324 mmol) was added to it. The mixture was stirred at reflux for 3.5h and cooled. The mixture was allowed to stand at room temperature overnight. Ethanol (100 ml) was added and the solid filtered, washed with ethanol and dried. The solid was next stirred with a saturated bicarbonate solution (200 ml) for 1h, diluted with water (500 ml) and filtered. The residue was washed with water and dried over phosphorus pentoxide to give 15.5 gm (66 % yield) of the product. ^{1}H-NMR (DMSO-d_6) d, 10.16, bs, (D_2O exchangeable), 1H, imidazole N-H; 8.29, s, 1H, H-8; 3.20, bs, 2H, N-H; 2.25, 2.18, 2s, 3H each, Ac-CH_3.

Example-3. Glycolysation Of Protected Bases With 3'-deoxysugar 7.

a: General procedure for pyrimidine bases (C, U and T)

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(Figure-2):

The pyrimidine base (13 mmole) was co-evaporated with dry toluene (2 x 20 ml). The base was then suspended in excess hexamethyldisilazane (50 ml) and ammonium sulfate (1.3 mmol) and heated for 2h till clear. Excess HMDS was evaporated in vacuo and the residue taken up in dry acetonitrile (20 ml) followed by the addition of sugar (13.3 mmol) and trimethylsilyltriflate (13.3 mmol) under a blanket of Argon. The reaction mixture was stirred for 12h at room temperature and poured into bicarbonate solution (100 ml). Extraction with dichloromethane (3 x 50 ml) gave the organic extract which was dried (sod. sulfate) and concentrated to give the crude nucleoside. Purification on a column of silica gel (30 parts of adsorbent for 1 gm crude nucleoside) gave the pure nucleoside in 85-87 % yield.

b: General procedure for purine bases (A and G) :

Identical procedure as above except silylation done with excess N,O-bis trimethylsilylacetamide (12 ml) and the reaction conducted in dry dichloroethane at 80°C for 8h. After hydrolysis the products were obtained in 65-87 % yield. Glycolysation with adenine gave N9 and N7 regioisomers in a ratio of 3:2.

Example-4. Deprotection Of 3'-Deoxynucleosides (Step-viii).

a: Complete deprotection:

The purified 3'-deoxy nucleosides (10 mmole) is dissolved in methanol (10 ml) and treated with conc. ammonium hydroxide at room temperature (overnight) or at 60°C for 48h (for guanosine). Solvent was removed to furnish the crude

nucleoside.

b: Partial depr tection:

The purified 3'-deoxy nucleosides were treated with a solution of dioxan:water:1NNaOH (3:1:1) in a ratio of 5 equivalents of base per nucleoside at room temperature for 4h. The pH is adjusted to 7 with 0.1N HCl and the solvents removed in vacuo. This procedure hydrolyzes the ester linkages and leaves base protections intact.

Example-5. Dimethoxytritylation Of Nucleosides (Step-xi).

The nucleosides (10 mmole) were co-evaporated with dry pyridine (2 x 50 ml) and resuspended in dry pyridine (21 ml). Dimethoxytrityl chloride (12 m mole) was added and the mixture stirred overnight at room temperature. Methanol was added (5 ml) and after stirring for 0.5h the reaction mixture was poured in bicarbonate solution (200 ml). The dimethoxytritylated nucleoside either separated out as a solid which was filtered and collected or the aqueous solution was extracted with dichloromethane (3 x 50 ml) and the organic phase dried and concentrated to give the product. The crude product was loaded on a column of silica gel (10 gm of silica gel/1gm nucleoside) and the impurities eluted with ether. The pure compound eluted with ethyl acetate was obtained in 70-80% yield.

N^4-benzoyl-5'-O-dimethoxytrityl-3'-deoxycytidine: ^{1}H-NMR ($CDCl_3$) d, 8.73, bs, (D_2O exchangeable), N-H; 8.45, d, 1H, H-6; 7.87, d, 1H, H-5; 7.63-7.16, m, 14H, Ar-H; 6.86, d, 4H, o-Ar-H to OCH_3, 5.79, s, 1H, H-1'; 4.70, m, 1H, H-2'; 4.51, bs, 1H, H-4'; 4.31, bs, (D_2O exchangeable), O-H; 3.80, s, 6H,

OCH_3; 3.56, 3.31, 2d, 2H, H-5'; 2.22, 2.06, 2m. 2H, H-2'.

5'-O-dimethoxytrityl-3'-deoxy-5-methyl uridine: ^{1}H-NMR ($CDCl_3$) d, 10.24, bs, (D_2O exchangeable), N-H; 7.80, s, 1H, H-6; 7.53-7.16, m, 9H, Ar-H; 6.83, d, 4H, o-Ar-H to OCH_3, 5.76, s, 1H, H-1'; 4.92, m, 1H, H-2'; 4.65, bs, 1H, H-4'; 4.51, bs, (D_2O exchangeable), O-H; 3.78, s, 6H, OCH_3; 3.57, 3.28, 2d, 2H, H-5'; 2.27, 2.02, 2m. 2H, H-2'; 1.41, s, 3H, C-CH_3.

N^2-(dimethylamino)methylene-5'-O-dimethoxytrityl-3'-deoxy-guanidine: ^{1}H-NMR (DMSO-d_6) d, 11.33, bs, (D_2O exchangeable), N-H; 8.54, s, 1H, H-8; 7.86, s, 1H, =C-H; 7.39-6.74, m, 13H, Ar-H; 5.85, s, 1H, H-1'; 5.66, bs, (D_2O exchangeable),1H, O-H; 4.60, m, 1H, H-2'; 4.44, bs, 1H, H-4'; 3.70, s, 6H, OCH_3; 3.11, 3.02, 2s, 3H each, N-CH_3; 3.45, m, 2H, H-5'; 2.27, 1.97, 2m. 2H, H-2'.

N^2-benzoyl-5'-O-dimethoxytrityl-3'-deoxyadenosine: ^{1}H-NMR ($CDCl_3$) d, 9.12, bs, (D_2O exchangeable), N-H; 8.79, s, 1H, H-8; 8.29, s, 1H, H-2; 7.59-7.20, m, 14H, Ar-H; 6.78, d, 4H, o-Ar-H to OCH3, 5.99, s, 1H, H-1'; 4.90, bs, (D_2O exchangeable),1H, O-H; 4.70, m, 1H, H-2'; 4.64, bs, 1H, H-4'; 3.77, s, 6H, OCH_3; 3.42, 3.27, m, 2H, H-5'; 2.34, 2.23, m. 2H, H-2'.

EXAMPLE-6: <u>Synthesis of 2'-5' oligonucleotides</u>

(2'-5')-3'-deoxyoligonucleotides were prepared via both the syringe techniques as well as the DNA synthesizer using standard phosphoramidite reagents and controlled pore glass RNA (lcaa-CPG-500 A°) solid support. The average coupling reaction yield was 98.0-99.5% as determined by absorbance of the dimethoxytrityl cation liberated on treatment with 3% dichloroacetic acid in methylene chloride. The

oligodeoxynucleotide was cleaved from the solid support (concentrated ammonium hydroxide, 4h, 25°C) and the protecting groups were removed (concentrated NH_4OH, 5h, 55°C). The ammonium hydroxide was evaporated and the crude product purified by HPLC ion-exchange chromatography (Dionex OMNI PAK (TM)) and desalted on a C18 SEP PAK (TM) cartridge (Millipore). By this protocol oligonucleotides of >95% purity were obtained.

EXAMPLE-7; Comparison Of thermal stability (T_m) Of 2'-5' homo-oligonucleotides with RNA and DNA targets.

To effectively inhibit gene expression in mammalian cells it is essential that an oligonucleotide recognize and bind tightly to its complementary sequence in the target nucleic acid. The affinity between two oligonucleotides can be determined by spectrophotometric methods, where absorbance versus temperature is measured for an equimolar mixture of complementary oligonucleotides (Wickstrom, E.; Tinoco, I. Jr. *Biopolmers* 1974, 13, 2367). Base stacking between complementary oligonucleotides is accompanied by a reduction in UV absorption (hypochromicity). When the temperature of the solution containing double-helical DNA (or RNA) is slowly raised, UV absorption increases suddenly at a certain temperature as the double helix dissociates. In this way it is possible to assess the hybrid stability from the melting temperature (Tm), or the temperature required for dissociation of half the duplex to single strand.

To assess the effects of the 3'-deoxy-(2'-5') internucleotide linkage on duplex stability the melting

temperatures were determined by hypochromicity at 260 nm from 10°C to 70°C at a concentration of approximately 4 μM of each strand and compared with the natural (3'-5') oligomer. When (2'-5')-3'-dA_8 was mixed with an equimolar concentration of poly U a monophasic helix-coil transition was observed upon heating the solution from 10°C to 70°C. The Tm of (2'-5')-3'dA_8/ poly U heteroduplex in 10mm sodium phosphate/pH 7.4 and 1.0m NaCl was 53°C compared to 54°C for the natural 3'-5' linked dAg/poly U heteroduplex. In control experiments, when (2'-5')-3'-dA_8 was mixed with poly C, poly A or alternating poly AU no hypochromicity was observed (data not shown). Furthermore, controls performed by Switzer (Hashimoto, H; Switzer, C. *J. Am. Chem. Soc.*, 1992, 114, 6255) where absorbance profiles versus temperature for pure (2'-5')-3'dA_{12} separately, exhibit a slight linear change in hyperchromicity consistent with reported UV absorbance behavior of deoxyoligoadenylates (Leng, M; Felsenfeld, G.J. Mol. Biol. 1966, 15, 455). This is consistent with our own control experiments which showed no evidence of self-association when (2'-5')-3'dAG was heated along. Our control and experimental results taken together demonstrate no possibility of purimic self-association leading to the profile that results from the mixture of (2'-5')-3'dAg and poly U. It is particularly noteworthy that (2'-5')-3'-dA_8 exhibited a barely detectable variation in hyperchromicity with complementary poly dT when heated from 10°C to 70°C indicating that (2'-5')oligo-3'-deoxynucleotides do not bind to single strand DNA. In contrast, dA_8 exhibits apparent hypochromicity

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when mixed with complementary poly dT (Tm=62°C), which is in agreement with reported values (Cassani, G.R.; Bollum, F.J. *Biochemistry* **1969**, 8, 3928). The Tm of (2'-5')-3'-dA_8 / poly U heteroduplex in 10 millimolar sodium phosphate buffer/pH 7.4 and 130 millimolar salt was 32°C with no observed duplex formation between (2'-5')-3'-dA_8/poly dT. In agreement with our findings, Brieslow also observed that a (2'-5')oligo-3'-deoxynucleotide of a mixed adenine-thymine sequence fails to strand associate to a complementary (3'-5') linked oligodeoxynucleotide suggesting that this represents a general failure of oligos constructed with this unique internucleotide linkage (Dougherty, J.P.; Rizzo, C.J.; Breslow, R. J. Am. Chem. Soc. 1992, 114, 6254). The remarkable selectivity for hybridization of (2'-5')-3'-dA_8 to complementary RNA rather than to DNA suggests that (2'-5')-3'-dA_8 and related mixed base and longer oligomers may serve as a unique class of RNA-specific antisense oligodeoxynucleotides. Oligonucleotide analogs which show some selective binding to RNA complements have been reported (Adams, A.D.; Petrie, C.R.; Meyer, R.B. *Nucleic Acids Res.* **1991**, 19, 3647) (Durand, M.; Maurizot, J.C.; Thuong, N.T.; Helene, C. *Nucleic Acids Res.* **1988**, 16, 5039). The highest RNA selectivity occurs with enantio-DNA, since no hypochromicity was observed when L-dA_6 was mixed with poly dT (Fujimori, S.; Shudo, K.; Hashimoto, Y. *J. Am. Chem. Soc.* **1990**, 112, 7436). However, the high preference of enantio DNA (L-dA_6) for complementary RNA also results in the formation of a much less stable heteroduplex (Tm=32.5°C) relative to natural

EXAMPLE-8: Comparison Of thermal stability (T_m) Of 2'-5' oligonucleotides with RNA and DNA targets.

DNA Target: TGT GTG GAA TTG TGA G

RNA Target: UGU GUG GAA UUG UGA G

Phosphodiester oligomers	DNA	RNA
1. 5'- CTC ACA ATT CCA CAC A-3'	58.5 °C	51 °C
2. 5'- CTC ACA ATT CCA CAC A-2'	-	40 °C

The values were determined in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride at 5 micro molar total strand concentration. 3'-5' connected phosphodiester 16 mer showed a Tm of 51 °C against the RNA target and 58.5 °C against the DNA target. In comparison, the 2'-5' oligonucleotide had a Tm of 40 °C with no duplex formation with complementary DNA target.

EXAMPLE-9: Comparison of thermal stabilities of 2'-5' and 3'-5' phosphorothioate oligonucleotides against complementary DNA and RNA targets

DNA Target: GGC GAG GAA CAC GGC GCG ATG CAG

RNA Target: GGC GAG GAA CAC GGC GCG AUG CAG

S-Oligomers	DNA	RNA
1. 5'-CATCGCGCCGTGTTCCTCGCC-3'	68.5 °C	60.5 °C
2. 5'-CATGGCGCCGTCTTCCTCGCC-3'	49.5 °C	42.0 °C
3. 5'-CATCGCGCCGTGTTCCTCGCC-2'	-	56.0 °C
4. 5'-CATGGCGCCGTCTTCCTCGCC-2'	-	35.5 °C

The values were determined in 10 mM sodium phosphate buffer (pH 7.0) containing 150 mM sodium chloride at 5 micro molar total strand concentration. A 21-mer 3'-5' connected phosphorothioate oligonucleotide had a melting temperature of 60.5 °C against

complementary RNA compared t 68.5 °C against DNA (entry 1). Same oligonucleotid sequence in 2'-5' connected m tif showed a Tm of 55 °C against RNA while showing n hybridization with complementary DNA (entry 3). When two mis-matches were incorporated in the 3'-5' oligomer a Tm drop of 18 °C was observed against the RNA target (entry 2) in comparison to th 19.5 °C drop in the Tm of corresponding 2'-5' oligomer.

EXAMPLE-10: **Thermal stabilities of chimeric 2'-5'/3'-5' oligonucleotides against complementary RNA target:**

RNA Target: GGC GAG GAA CAC GGC GCG AUG CAG

	oligonucleotideS	RNA
1.	(PS) 5'-CAT CGC GCC GTG TTC CTC GCC-2'	56 °C
2.	(PO) 5'-CAT CGC Gcc gtg ttc CTC GCC-2'	60 °C
3.	(PS) 5'-CAT CGC Gcc gtg ttc CTC GCC-2'	58 °C
4.	(PS) 5'-CAT GGC GCC GTC TTC CTC GCC-3'	42 °C.
5.	(PS) 5'-CAT GGC Gcc gtc ttC CTC GCC-2'	38 °C

Capital letters denote chain length that is 2'-5' connected whil small letters denote 3'-5' connection. Thus, incorporation of 3'-5' motif in 2'-5' connected oligonucleotides in both PO as well as PS oligonucleotides gives a slight increase in the hybrid stability against complementary RNA with sharp drop in duplex stability when mis-matches are introduced (entry 5 versus entry 3)

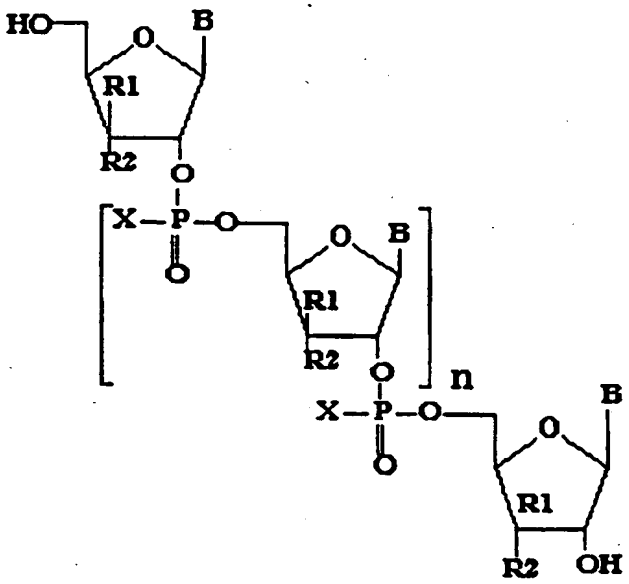
What is claimed is:

1. A method of selectively hybridizing an oligonucle tide to RNA comprising hybridizing in a sequence specific manner at least one complementary oligonucleotide to said RNA, said oligonucleotide comprising at least one 2'-5' internucleotide linkage.

2. The method of claim 1 wherein said oligonucleotide is from about 8 to 75 nucleotides in length.

3. The method of claim 1 wherein said oligonucleotide is a methylphosphonate.

4. The method of claim 1 wherein said oligonucleotide has the formula



wherein B is selected from the group consisting of adenine, guanine, thymine, cytosine, 5 methylcytosine, uracil and inosine; R_1 and R_2 are each independently selected from the group consisting of hydrogen, hydroxyl, alkyl, aryl, alkoxy, alkyoxy, aryloxy, and azido; X is selected from the group consisting of

oxygen, sulfur, alkyl, allyl, aryl ph sphonate having fr m 1 t about 20 carbon atoms, alkoxy, alloxy, aryloxy phosphotriester having from one to about 20 carbon atoms, alkylamine, allylamine, arylamine ph sphoramidate having from 1 to about 20 carbon atoms, S-alkyl, S-allyl and S-aryl phosphorothioate having from one to about 20 carbon atoms and at least one nucleoside unit of the oligonucleotide is connected by a 2'-5' internucleotide linkage. n can be from 6 to 73 nucleotides long.

5. The method of claim 1 wherein said oligonucleotide is substituted by CH_2, NH or S at at least one of the 5' and 2' oxygen atoms of the phosphate backbone.

6. The method of claim 1 wherein said oligonucleotide is a phosphorothioate.

7. The method of claim 1 wherein said oligonucleotide is a phosphodiester.

8. The method of claim 3, 6 or 7 wherein the oligonucleotide is a phosphorothioate and represented in formula of claim 4 as X = sulfur or a phosphodiester represented in formula of claim 4 as X = oxygen or a methylphosphonate represented in formula of claim 4 as X = CH_3 wherein R_1 and R_2 are each independently selected from the group consisting of hydrogen, hydroxy, methoxy, allyloxy, azido and amino.

9. The method of claim 1 wherein said oligonucleotide is chemically modified at at least one site to enhance its permeability into cells.

10. The method of claim 1 wherein said oligonucleotide is chemically modified at at least one site to increase the binding strength of hybridization to complementary RNA.

11. The method of claim 1, further comprising at least one 3'-5' linkage.

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12. The method of claim 11 wherein at least one 3'-5' linkage is a phosphodiester linkage.

13. The method of claim 11 wherein at least one 3'-5' linkage is a phosphorothioate linkage.

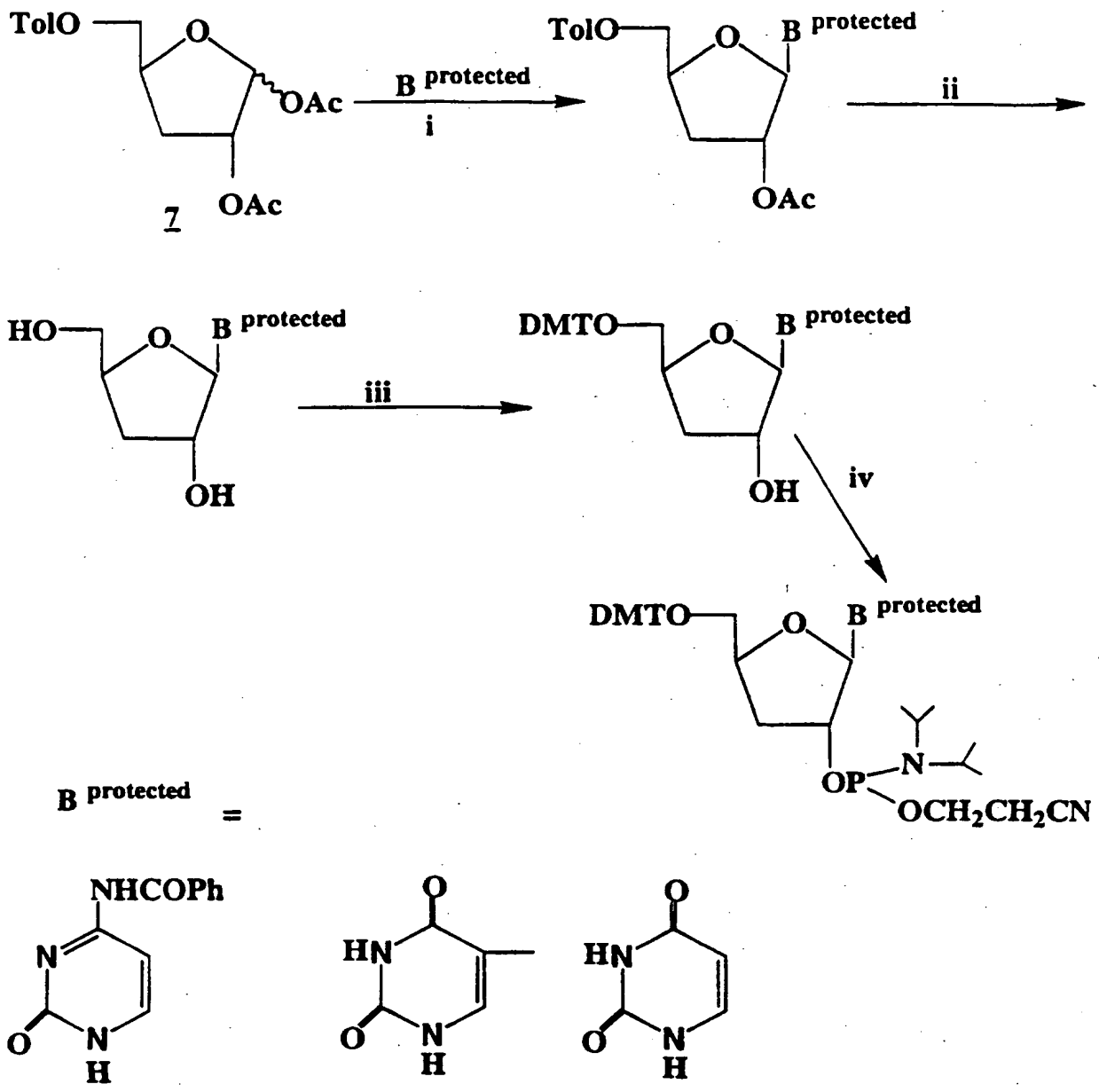

Figure-2. Synthesis of pyrimidine building blocks: i:HMDS/Ammonium sulfate, then 7 and TMSOTf/CH_3CN, 87-89%; ii:IN NaOH/Di xane/H_2O (1:3:1) 4h; or NH_4OH, 12h, RT, 90-92%; iii: DMTCl/Pyd, 8h, 84-90%; iv:DIPEA/ $[(CH_3)_2CH]_2NP(Cl)OCH_2CH_2CN$; 73-80%.

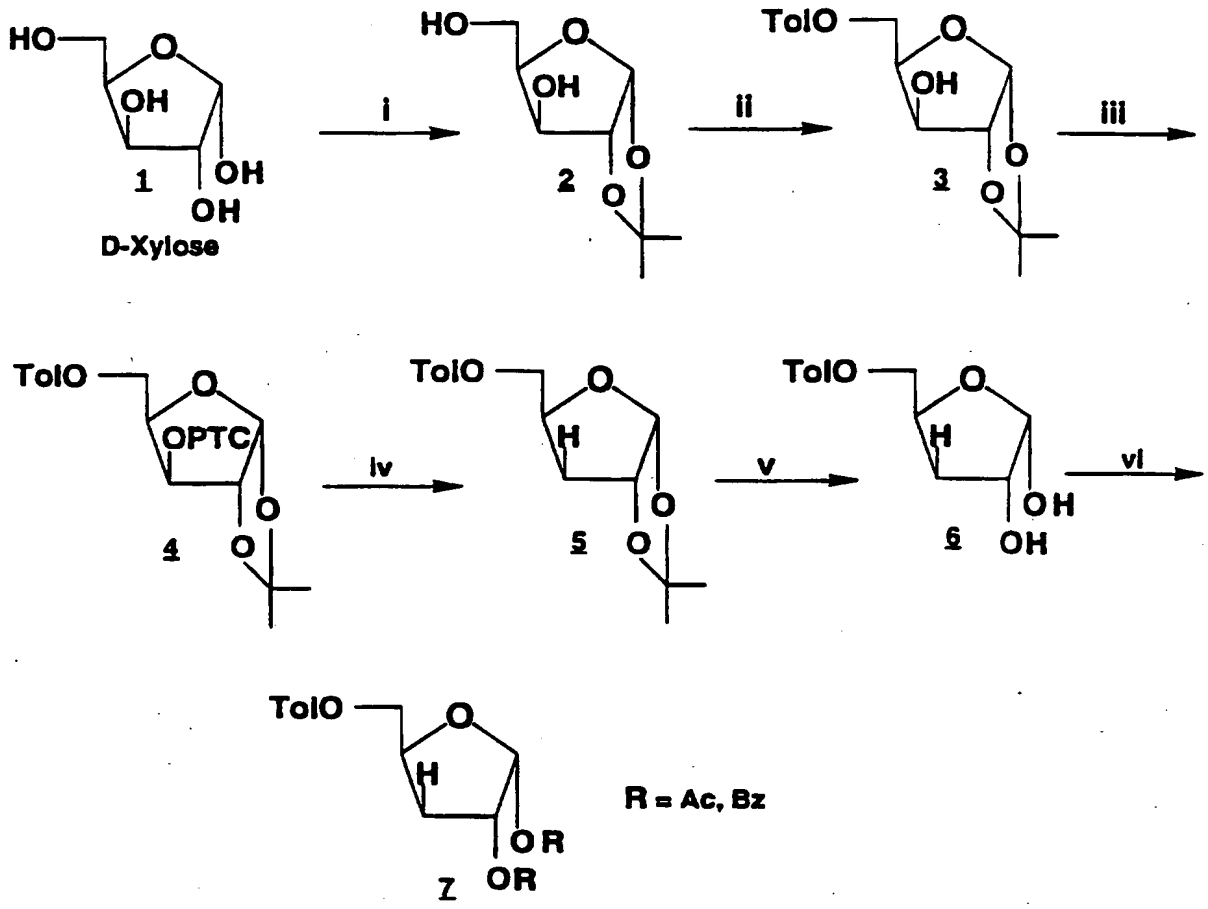

Figure-1. Synthesis Of the 3'-deoxy sugar: i: Acetone/H_2SO_4, 80%; ii: Toluyl Chloride/pyridine, 66%; iii: Phenoxythiocarbonyl Chloride/DMAP; iv: Bu_3SnH/ALBN, 81% from iii; v, HCOOH , 60°C, 5h; vi: $(RO)_2O$/Pyridine, 65% from V.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/11161

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 19/00, 21/00, 21/02, 21/04
US CL :435/6; 536/22.1, 23.1, 24.3, 25.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/22.1, 23.1, 24.3, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN
search terms: nucleic acid, DNA, RNA, hybridiz, antisense, modified nucleotides

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,470,967 A (HUIE ET AL.) 28 November 1995, see entire document.	1-13
X	EP 0 552 766 A2 (HOECHST AG) 28 July 1993, see entire document.	1-13
X	EP 0 552 767 A2 (HOECHST AG) 28 July 1993, see entire document.	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 23 SEPTEMBER 1996	Date of mailing of the international search report 06 NOV 1996
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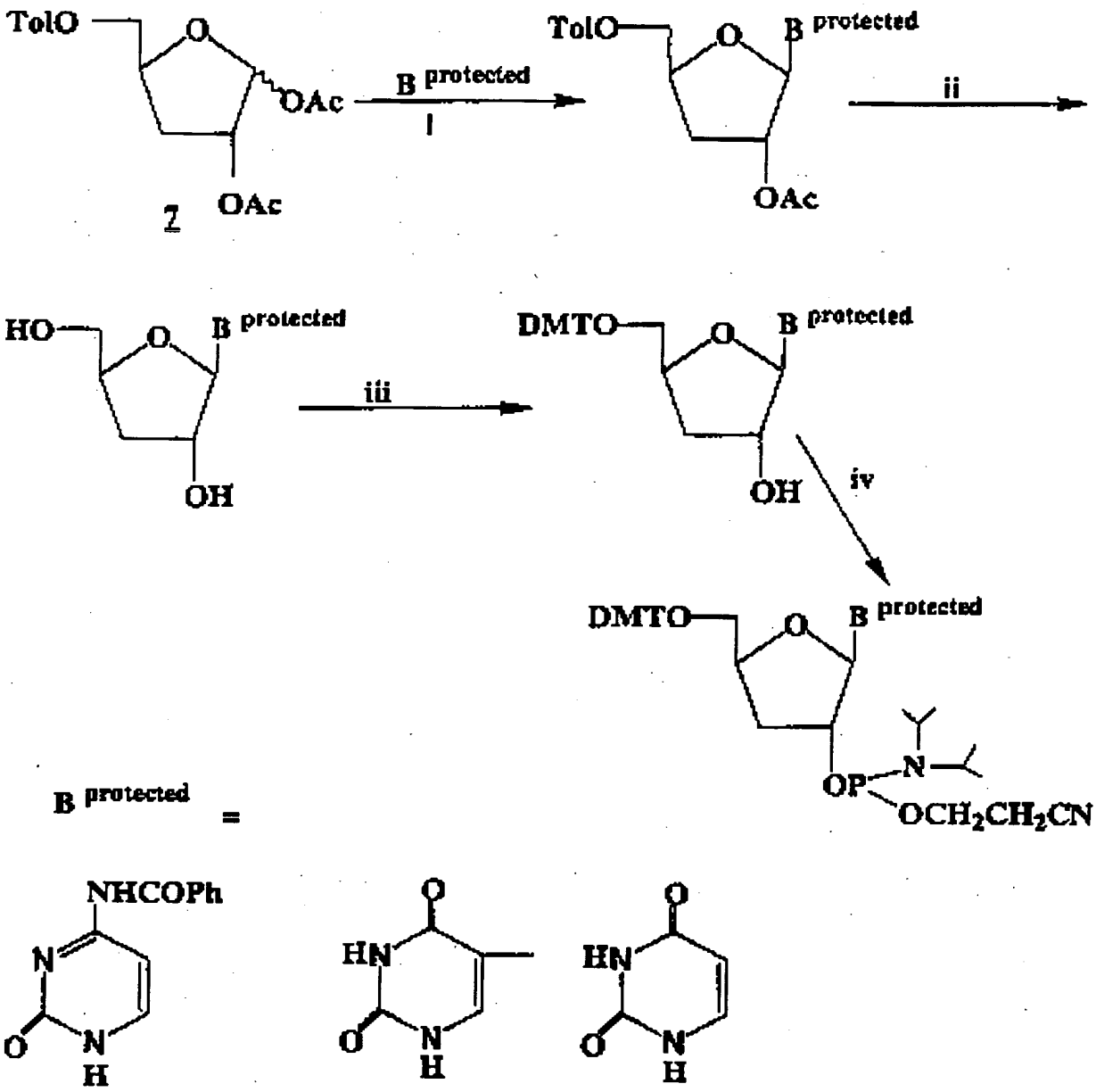

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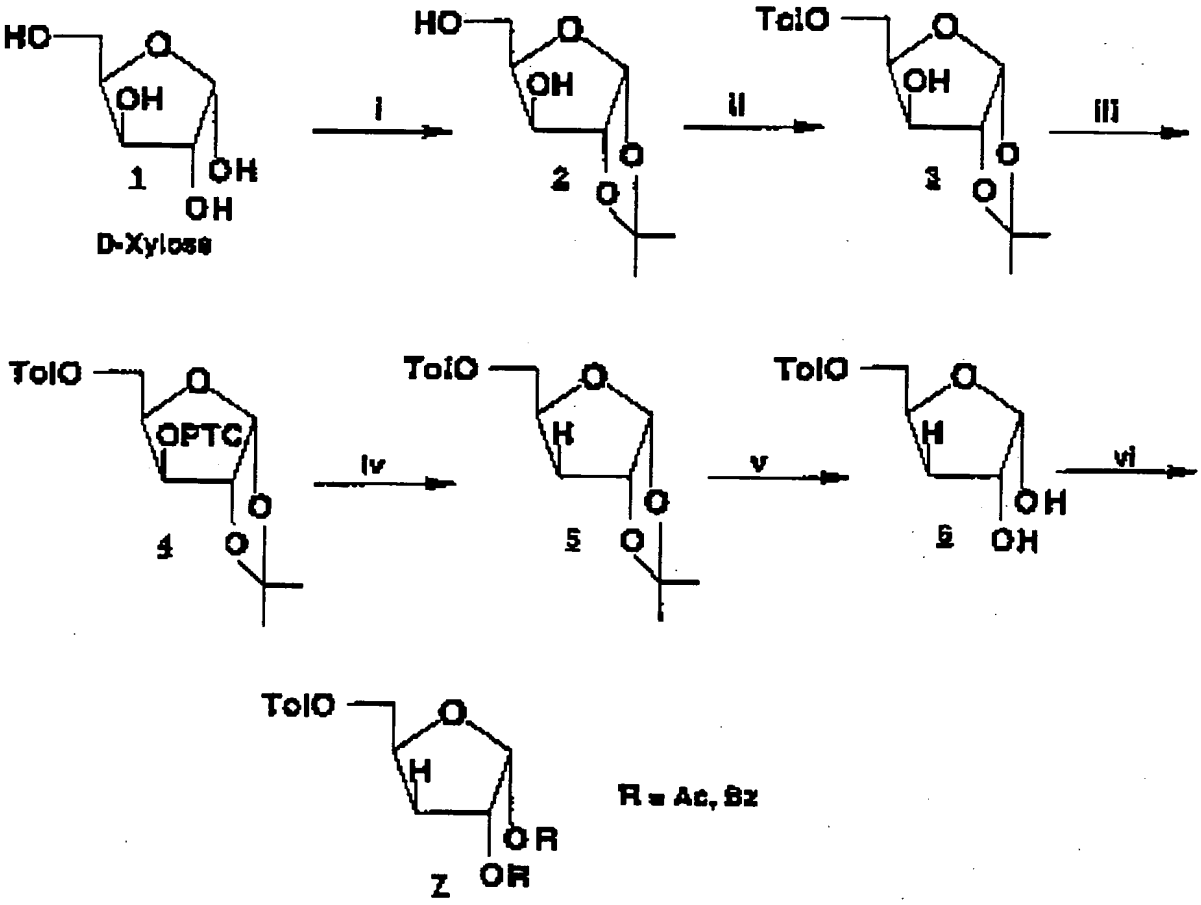

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